IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Kwon, Byoung	Examiner: C. Kaufman
Serial No.:	08/955,572	Group Art Unit: 1646
Filed:	October 22, 1997	Docket: 740.013US2
Title:	NEW RECEPTOR AND RELATED PRODUCTS AND METHODS	

SUPPLEMENTAL DECLARATION UNDER 37 C.F.R. § 1.131(b)

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Byoung Kwon declare and say as follows:

1. I am the named inventor of the subject matter claimed in the above-identified patent application, U.S. application Serial No. 08/955,572, filed on October 22, 1997. As amended, the above-identified application claims the benefit of the filing date of U.S. application Serial No. 08/122,796.

2. I received a Certificate in 1968, a D.D.S. in 1972, and a M.S. in Microbiology in 1974, from Seoul National University, Seoul, Korea. In 1981, I received a Ph.D. in microbiology from the Medical College of Georgia, Augusta, Georgia. From 1981-1984, I was a postdoctoral fellow in the Department of Human Genetics at Yale University School of Medicine, New Haven, Connecticut. I was the Head of Medical Genetics at the Gutherie Research Institute, Sayre, Pennsylvania, from 1984-1988. From 1988-1993, I was an Associate Professor in the Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, Indiana. I am currently a Professor in that same Department. I have authored or co-authored over 100 papers, primarily in the areas of the molecular basis for pigmentation and the identification and characterization of molecules involved in lymphocyte activation and proliferation.

3. I have reviewed the Schwarz et al. document (Genbank Accession No: L12964) cited by the Examiner in the Office Action dated April 22, 1998, the Amendment and Declaration filed on October 26, 1998, and the Rule 116 Response filed herewith, and make this

Declaration in support of the patentability of the claims of U.S. patent application Serial No. 08/955,572, as amended in the Amendment filed on October 26, 1998.

4. Prior to the April 22, 1993 publication date of Schwarz et al., I had isolated and purified a portion of a human 4-1BB gene and thereafter proceeded diligently to characterize the full length gene.

5. Schwarz et al. disclose the nucleotide sequence and inferred amino acid sequence of a human cDNA termed ILA. The nucleotide sequence encodes a polypeptide that has a single amino acid substitution relative to SEQ ID NO:2 of the present application.

6. Exhibits A, B, C, and D, attached hereto and incorporated by reference herein, are submitted as factual evidence of conception of the invention in the United States prior to the effective date of the above-mentioned reference coupled with due diligence from conception in the United States to constructive reduction to practice, as evidenced by the filing of U.S. application Serial No. 08/122,796, which is the parent application to the above-identified divisional application.

7. Exhibits A, B and C are submitted as factual evidence that the invention was conceived in the United States prior to the effective date of Schwarz et al. Exhibit A is a photocopy of certain pages, two of which are pages corresponding to Figures 2A and 2B, from U.S. application Serial No. 08/012,269, an application of which I am the sole inventor. The application discloses the nucleotide sequence and inferred amino acid sequence of murine 4-1BB (page 17, and Figures 2A and 2B). A method of isolating the human homolog of murine 4-1BB is disclosed at page 24. The preparation of soluble murine 4-1BB and a murine 4-1BB fusion protein is described at pages 29 and 70, respectively.

8. Exhibit B and Exhibit C are each a photocopy of an autoradiogram. Various combinations of degenerate primers (such as those described at pages 14-15 of the present specification) and human lymphocytic RNA were employed in a reverse transcriptase-

polymerase chain reaction to obtain amplification products that corresponded to the human homolog of murine 4-1BB. The products were fractionated on agarose gels, individual DNA bands were cut out and the DNA eluted therefrom. The eluted DNAs were subjected to gel electrophoresis, transferred to a membrane filter and the filter probed with radiolabeled murine 4-1BB DNA under low stringency conditions, the results of which were recorded on an autoradiogram (Exhibit B). The DNA in the hybridizing band in lane 7 of Exhibit B was cloned and inserted into a vector which was then introduced to host cells. DNA in the vector-transformed host cells was transferred to a membrane filter and the filter probed with radiolabeled murine 4-1BB DNA, the results of which were recorded on an autoradiogram (Exhibit C). The experiments which produced Exhibits B and C were performed in my laboratory at Indiana University, Indianapolis, Indiana, USA. Exhibit B, and Exhibit C (date masked out), are dated prior to April 22, 1993.

9. Exhibit D is included as factual evidence that the invention as conceived was diligently pursued from a time preceding the effective date of Schwarz et al. to its constructive reduction to practice. Exhibit D is a photocopy of the filing receipt for Serial No. 08/122,796, which is the parent application to the above-identified divisional application. Exhibit D demonstrates that the invention disclosed in Exhibits A, B and C was diligently pursued from a time before the effective date of Schwarz et al., i.e., April 22, 1993, to a time approximately five months after the effective date of Schwarz et al., at which time the invention was constructively reduced to practice.

10. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: 1-29-99 By: Byoung Kwon

Byoung Kwon

Exhibit A

sequence of 4-1BB revealed a single long open reading frame, beginning with the ATG codon at nucleotide residues 1-3 (Fig. 2b.). This reading frame codes for a polypeptide of 256 amino acids with a molecular weight of 27,587. The assigned ATG is preceded by an in-frame termination codon TGA (nucleotide residues -12 to 9). The sequence flanking the assigned ATG (nucleotide residues -5 to 4) is a favored sequence for eukaryotic initiation sites (consensus; CCG/ACCATGG) described by Kozak (30). In fact, 8 out of 9 consensus sequences were identical to the sequences flanking to the assigned initiation codon. The codon specifying carboxy-terminal leucine is followed by the translational termination codon TGA (nucleotide residues 659-771). 4-1BB contains 1434 nucleotides of 3'-untranslated region which did not extend as far as polyadenylation signal nor the poly $(A)^+$ tail.

Figure 2 shows the nucleotide sequence and the deduced amino acid sequence of 4-1BB. The nucleotides of the message strand are numbered in the 5' to 3' direction and numbers are shown on both sides of the sequence. Nucleotide residue 1 is the A of the initiation codon ATG, and the nucleotides on the 5' side of residue 1 are indicated by negative numbers. The predicted amino acid sequence is shown below the nucleotide sequence. Putative signal peptide is underlined. The potential asparagine-linked glycosylation sites are underlined. Potential polyadenylation signal is boxed. Stop codon is indicated by (---). Cysteine residues are highlighted by (·). An unusual feature of 4-1BB sequence is that there is a potential polyadenylation signal of AATAAA at nucleotides 1158-1163 (Fig. 2b boxed). It was believed that this signal was functional because this gene produces at least two different sizes of mRNA. We believe that this signal of AATAAA at nucleotides 1158-1163 (Fig. 2b boxed). We believe that this signal is functional because this gene produces at least two different sizes of mRNA.

Isolation of human lymphokines and receptors homologous to L2G25B and 4-1BB. L2G25B and 4-1BB cDNA may be used as probes to isolate human lymphokines or receptors homologous to these type clones. Each cDNA will be radio-labeled and hybridized to human genomic DNA blot under various stringency and washing conditions using standard laboratory techniques known to those skilled in the art.

The species difference in nucleotide sequences between human and mouse will determine the degree of homology by clone hybridization experiments. On the determination of the optimal hybridization and washing conditions under which the probes detect a signal in the human genomic DNA blot, then a human genomic library in lambda vector may be screened with radio labeled L2G25B and 4-1BB. The hybridizing human clones may then be isolated and the nucleotide sequences determined.

The genomic human clone corresponding to mouse clone L2G25 and 4-1BB may then be used as a probe to survey human T cells which express mRNA by RNA blot analysis. When the human T cells which express the RNA homologous to L2G25B and 4-1BB are discovered and isolated, the RNA may then be used to construct a cDNA library. Then the cDNA library may be screened with the human genomic clone corresponding to L2G25B and 4-1BB and isolate the human cDNA clones corresponding to L2G25B and 4-1BB.

Construction of the Expression Plasmid of Truncated 4-1BB. The putative extracellular domain of 4-1BB cDNA was amplified by polymerase chain reaction (PCR) (100). An Xhol site was created at the 5' end of the forward primer and a stop codon, (TAA), and an Eco R1 site were created in the reverse primer. The PCR product was digested with Xhol and Eco R1 and the ~0.6 kb fragment was purified. The Xhol-Eco R1 fragment (P4-1BBs) was inserted into the PXM vector (101).

Production of the Recombinant Truncated 4-1BB Protein. COS-1 cells were grown to 30-50% confluency and were transfected with the truncated 4-1BB in the PXM vector using the DEAE dextran method (102). Forty-four hours post transfection, the culture medium was replaced with serum-free medium (Opti MEM, Gibco Laboratories, Grand Island, NY). The culture medium was harvested twice every 24 hrs. The proteins in the conditioned medium were precipitated with 4 volumes of acetone at -20°C and resuspended in a mixture consisting of a chromatography buffer (50 mM Tris, pH 7.4, 0.15 M NaCl and 0.05% Tween -80), 5M urea and 1% β-mercaptoethanol. After the removal of undissolved particles by brief microcentrifugation, the supernatant was subjected to Sephadex G-200 chromatography. The fractions that were reactive with rabbit anti-4-1BB-0 antiserum in Western blot analysis, were pooled. The truncated, thus soluble 4-1BB protein (4-1BBPs) was further enriched through fractionation with Q-Separose column (Pharmacia Fine Chemicals) with a linear gradient of Nacl from 0.0 to 1.0M. The amino-terminal sequence of 4-1BBPs was determined by an automatic peptide sequencer PI 2090 (Proton Instrument, Tarzana, CA) after the protein was transferred to Immobilon-p (Millpore, Bedford, MA).

Production of the 4-1BB-AP fusion protein. The 5' portion of the 4-1BB cDNA including sequences encoding the original signal peptide and the entire extracellular domain, was amplified by the polymerase chain reaction (PCR) (99). For correctly oriented cloning, a Hind III site on the 5' end of the forward primer and a Bgl II site on the 5'end of the reverse primer were created. The Hind III-Bgl II 4-1BB fragment was inserted into the mammalian expression vector AP-tag-1, upstream of the coding sequence for human placental alkaline phosphatase (Ap) (113). (The AP-tag-1 vector was a kind gift of Dr. John Flanagan, Harvard University, Cambridge, MA). Sequence analysis of the fusion region confirmed that the 4-1BB and AP sequences were joined in frame. The 4-1BB-AP plasmid, linearized with Cla I, was cotransfected with the linearized-selectable marker plasmid, pSV7neo, by the calcium phosphate coprecipitation method. After selection in 500 μg/ml G418, resistant colonies were picked and expanded. Clones were subsequently screened for secretion by assaying for AP activity. Supernatant from one clone, 4-1BB-AP-2, which produced the 4-1BB-AP fusion protein, showed high levels of alkaline phosphatase activity; 738 OD units/hr/ml. When determining total 4-1BB-AP or AP activity, serial dilutions were performed so that AP activity was measured at non-saturating levels. The 4-1BB-AP or AP was then diluted accordingly so equivalent levels of 4-1BB-AP or AP activity were added to each sample. DMEM-CM containing purified human placental AP (Sigma, St. Louis MO) was utilized as a background control in all experiments.

Fig. 2B

781 CTAGGAGATG TGTGGGCCGA AACCGAGAAG CACTAGGACC CCACCATCCT GTGGAACAGC ACAAGCAACC 850

851 CCACCACCCT GTTCTTACAC ATCATCCTAG ATGATGTGTG GGCGCGCACC TCATCCAAGT CTCTTCTAAC 920

921 GCTAACATAT TTGTCTTTAC CTTTTTTAAA TCTTTTTTTA AATTTAAATT TTATGTGTGT GAGTGTTTTG 990

991 CCTGCCTGTA TGCACACGTG TGTGTGTGTG TGTGTGTGAC ACTCCTGATG CCTGAGGAGG TCAGAAGAGA 1060

1061 AAGGGTTGGT TGCATAAGAA CTGGAGTTAT GGATGGCTGT GAGCCGGnnn GATAGGTCGG GACGGAGACC 1130

1131 TGTCTTCTTA TTTTAACGTG ACTGTATAAT AAAAAAAAAA TGATATTTCG GGAATTGTAG AGATTGTCCT 1200

1201 GACACCCTTC TAGTTAATGA TCTAAGAGGA ATTGTTGATA CGTAGTATAC TGTATATGTG TATGTATATG 1270

1271 TATATGTATA TATAAGACTC TTTTACTGTC AAAGTCAACC TAGAGTGTCT GGTTACCAGG TCAATTTTAT 1340

1341 TGGACATTTT ACGTCACACA CACACACACA CACACACACA CACGTTTATA CTACGTACTG TATCGGTAT 1410

1411 TCTACGTCAT ATAATGGGAT AGGGTAAAAG GAAACCAAAG AGTGAGTGAT ATTATTGTGGA GGTGACAGA 1480

1481 CTACCCCTTC TGGGTACGTA GGGACAGACC TCCTTCGGAC TGTCTAAAAC TCCCCTTAGA AGTCTCGTCA 1550

1551 AGTTCCCGGA CGAAGAGGAC AGAGGAGACA CAGTCCGAAA AGTTATTTTT CCGGCAAATC CTTTCCCTGT 1620

1621 TTCGTGACAC TCCACCCCTT GTGGACACTT GAGTGTCATC CTTGCGCCGG AAGGTCAGGT GGTACCCGTC 1690

1691 TGTAGGGGCG GGGAGACAGA GCCGCGGGGG AGCTACGAGA ATCGACTCAC AGGGCGCCCC GGGCTTCGCA 1760

1761 AATGAAACTT TTTTAATCTC ACAAGTTTCG TCCGGGCTCG GCGGACCTAT GGCGTCGATC CTTATTACCT 1830

1831 TATCCTGGCG CCAAGATAAA ACAACCAAAA GCCTTGACTC CGGTACTAAT TCTCCCTGCC GGCCCCCGTA 1900

1901 AGCATAACGC GGCGATCTCC ACTTTAAGAA CCTGGCCGCG TTCTGCCTGG TCTCGCTTTC GTAAACGGTT 1970

1971 CTTACAAAAG TAATTAGTTC TTGCTTTCAG CCTCCAAGCT TCTGCTAGTC TATGGCAGCA TCAAGGCTGG 2040

2041 TATTTGCTAC GGCTGACCGC TACGCCGCCG CAATAAGGGT ACTGGGCGGC CCGTCGAAGG CCCTTTGGTT 2110

2111 TCAGAAACCC AAGGCCCCCC TCATACCAAC GTTTCGACTT TGATTCTTGC CGGTACGTGG TGGTGGGTGC 2180

2181 CTTAGCTCTT TCTCGATAGT TAG AC

Fig. 2A

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-145                                                                ATGTC

-140  CATGAACTGC TGAGTGGATA AACAGCACGG GATATCTCTG TCTAAAGGAA TATTACTACA CCAGGAAAAG

 -70  GACACATTCG ACAACAGGAA AGGAGCCTGT CACAGAAAAC CACAGTGTCC TGTGCATGTG ACATTTCGCC

   1  ATG GGA AAC AAC TGT TAC AAC GTG GTG GTC ATT GTG CTG CTG CTA GTG GGC TGT GAG AAG   60
   1  Met Gly Asn Asn Cys Tyr Asn Val Val Val Ile Val Leu Leu Leu Val Gly Cys Glu Lys   20

  61  GTG GGA GCC GTG CAG AAC TCC TGT GAT AAC TGT CAG CCT GGT ACT TTC TGC AGA AAA TAC  120
  21  Val Gly Ala Val Gln Asn Ser Cys Asp Asn Cys Gln Pro Gly Thr Phe Cys Arg Lys Tyr   40
                 ↑                    •           •                     •

 121  AAT CCA GTC TGC AAG AGC TGC CCT CCA AGT ACC TTC TCC AGC ATA GGT GGA CAG CCG AAC  180
  41  Asn Pro Val Cys Lys Ser Cys Pro Pro Ser Thr Phe Ser Ser Ile Gly Gly Gln Pro Asn   60
                    •           •

 181  TGT AAC ATC TGC AGA GTG TGT GCA GGC TAT TTC AGG TTC AAG AAG TTT TGC TCC TCT ACC  240
  61  Cys Asn Ile Cys Arg Val Cys Ala Gly Tyr Phe Arg Phe Lys Lys Phe Cys Ser Ser Thr   80
       •           •           •                                     •

 241  CAC AAC GCG GAG TGT GAG TGC ATT GAA GGA TTC CAT TGC TTG GGG CCA CAG TGC ACC AGA  300
  81  His Asn Ala Glu Cys Glu Cys Ile Glu Gly Phe His Cys Leu Gly Pro Gln Cys Thr Arg  100
                       •       •                   •                       •

 301  TGT GAA AAG GAC TGC AGG CCT GGC CAG GAG CTA ACG AAG CAG GGT TGC AAA ACC TGT AGC  360
 101  Cys Glu Lys Asp Cys Arg Pro Gly Gln Glu Leu Thr Lys Gln Gly Cys Lys Thr Cys Ser  120
       •               •                                       •           •

 361  TTG GGA ACA TTT AAT GAC CAG AAC GGT ACT GGC GTC TGT CGA CCC TGG ACG AAC TGC TCT  420
 121  Leu Gly Thr Phe Asn Asp Gln Asn Gly Thr Gly Val Cys Arg Pro Trp Thr Asn Cys Ser  140
                                                       •                   •

 421  CTA GAC GGA AGG TCT GTG CTT AAG ACC GGG ACC ACG GAG AAG GAC GTG GTG TGT GGA CCC  480
 141  Leu Asp Gly Arg Ser Val Leu Lys Thr Gly Thr Thr Glu Lys Asp Val Val Cys Gly Pro  160
                                                                           •

 481  CCT GTG GTG AGC TTC TCT CCC AGT ACC ACC ATT TCT GTG ACT CCA GAG GGA GGA CCA GGA  540
 161  Pro Val Val Ser Phe Ser Pro Ser Thr Thr Ile Ser Val Thr Pro Glu Gly Gly Pro Gly  180

 541  GGG CAC TCC TTG CAG GTC CTT ACC TTG TTC CTG GCG CTG ACA TCG GCT TTG CTG CTG GCC  600
 181  Gly His Ser Leu Gln Val Leu Thr Leu Phe Leu Ala Leu Thr Ser Ala Leu Leu Leu Ala  200

 601  CTG ATC TTC ATT ACT CTC CTG TTC TCT GTG CTC AAA TGG ATC AGG AAA AAA TTC CCC CAC  660
 201  Leu Ile Phe Ile Thr Leu Leu Phe Ser Val Leu Lys Trp Ile Arg Lys Lys Phe Pro His  220

 661  ATA TTC AAG CAA CCA TTT AAG AAG ACC ACT GGA GCA GCT CAA GAG GAA GAT GCT TGT AGC  720
 221  Ile Phe Lys Gln Pro Phe Lys Lys Thr Thr Gly Ala Ala Gln Glu Glu Asp Ala Cys Ser  240
                                                                           •

 721  TGC CGA TGT CCA CAG GAA GAA GAA GGA GGA GGA GGA GGC TAT GAG CTG TGA TGTACTATC   780
 241  Cys Arg Cys Pro Gln Glu Glu Glu Gly Gly Gly Gly Gly Tyr Glu Leu ---
       •       •
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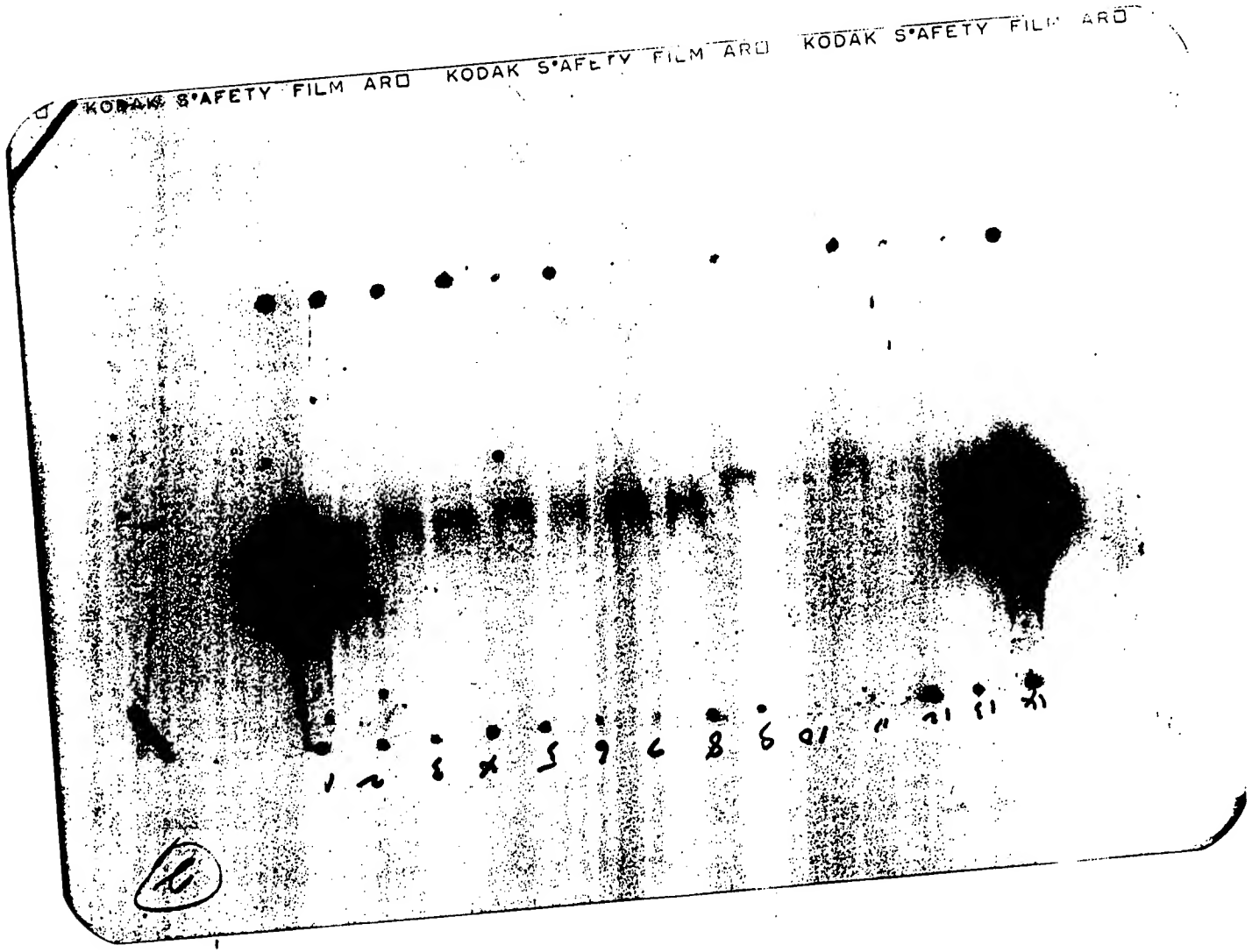
Exhibit B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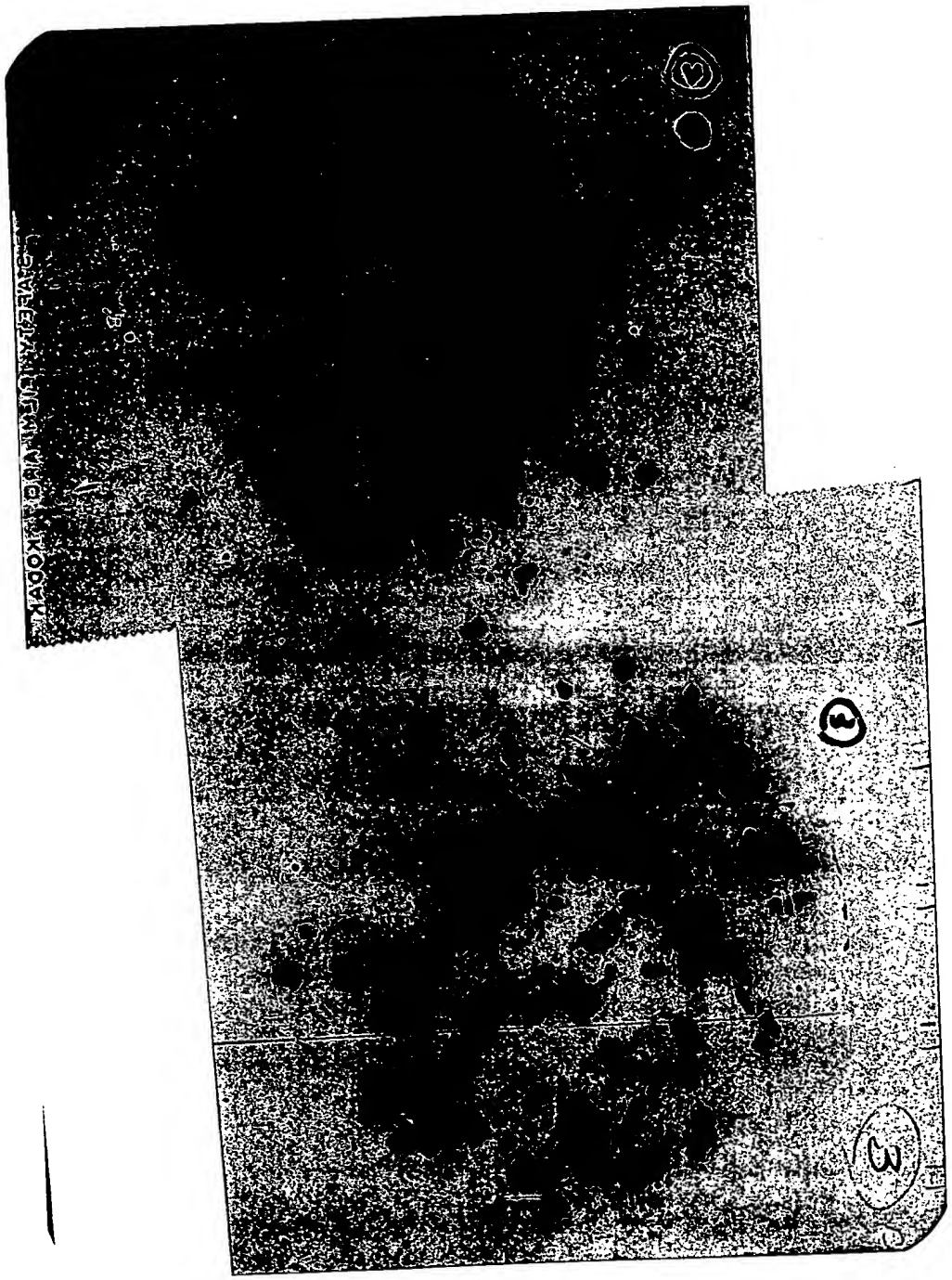

EXHIBIT C

PTO-103X
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APPLICATION NUMBER	FILING DATE	GRP ART UNIT	FIL FEE REC'D	ATTORNEY DOCKET NO.	DRWGS	TOT CL	IND CL
08/122,796	09/16/93	1813	$540.00		5	18	8

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Applicant(s) BYOUNG S. KWON, CARMEL, IN.

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THIS APPLN IS A CIP OF 08/012,269 02/01/93 740.9
WHICH IS A CIP OF 07/922,996 07/30/92 740.8
WHICH IS A CIP OF 07/267,577 11/07/88 ABN 740.6

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TITLE
RECEPTOR AND RELATED PRODUCTS AND METHODS

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EXHIBIT
D

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